

## The Relationship between Leptin, Adiponectin, Resistin and FoxP3<sup>+</sup> Treg cells in Patients with Severe Asthma

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### Abstract

**Objective:** The aim of this study was to evaluate the relationship between selected adipocytokines and Foxp3<sup>+</sup> Treg cells in patients with asthma.

**Methods:** The study included fifty-seven patients with asthma (30 with severe and 27 with mild to moderate asthma). The control group comprised 25 healthy volunteers. Asthma was diagnosed in accordance with GINA 2014 recommendation. The phenotype of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>Foxp3<sup>+</sup> cells was evaluated by multicolor flow cytometry. The concentrations of adipocytokine were determined by immunoenzymatic method (ELISA).

**Results:** The percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Treg cells expressing Foxp3<sup>+</sup> in the patients with severe (female 76.5% (66.1-85.2), male 67% (43.5-81.2)), were significantly lower than that of mild-to-moderate asthma (female 91.6% (78.8-94.5), male 84.5% (77.6-89.4)) and healthy controls (female 89.3% (76.6-94.6), male 94.5% (84.4-95.7)). We did not observed significant correlations between leptin or leptin receptor or adiponectin or resistin, concentration and MFC ratio. No significant correlations were observed between adipocytokine concentration and the percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup>.

**Conclusions:** The relationship between leptin, adiponectin, resistin and percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup> in patients with severe asthma was not observed.

**Keywords:** Adipocytokines; Asthma; Foxp3<sup>+</sup>; Treg cells

### Introduction

Asthma is a chronic inflammatory disorder of the airways. Mild and moderate form of the disease is effectively controlled with conventional therapy. Severe asthma is a particular clinical problem, in which glucocorticoid resistance can occur [1].

Much data focuses on the association between inflammation and adipose tissue. This inflammatory state is related to adipocytokines produced by adipocytes [2]. The role of adipocytokines in immunological response is known. Leptin has many proinflammatory activities on variety of cells such as CD4<sup>+</sup> T cells. This protein induces the proliferation of T lymphocytes, production of cytokines, mainly interleukin IL-1, IL-12, tumor necrosis factor (TNF)-α [3].

In opposite to leptin, adiponectin is an important anti-inflammatory protein. Adiponectin inhibits inflammatory gene expression such as IL-6 *via* the modulating nuclear factor (NF-κβ) and extracellular signal-regulated kinase activation. These adipocytokines enhances expression of anti-inflammatory genes, including the IL-10 [4,5].

Several reports have demonstrated the relationship between resistin and inflammation. Resistin increase the production of pro-inflammatory cytokines such as TNF-α and interleukin 12 which are important for T cell development [6,7].

The subpopulation CD4<sup>+</sup> T regulatory cells are characterized by constitutive expression of the transcription factor FoxP3. FoxP3 is needed to maintain the suppressive activity of Tregs in human. However, CD25<sup>high</sup> and CD127<sup>low</sup> can be used as markers in the identification of Tregs subpopulation [8,9]. CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>Foxp3<sup>+</sup> Tregs plays a role in maintaining appropriate immunological response in allergic diseases such as asthma [10,11].

Recent data suggest that both adipocytokines (leptin, adiponectin and resistin) and CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>Foxp3<sup>+</sup> Treg cells play a significant role in asthma inflammation. The results of EFUMOSA group showed that severe asthma is frequent in women with higher body fat [12]. In this study we focused on the relationship between selected adipocytokines and CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Treg cells expressing Foxp3<sup>+</sup> in women and men with severe asthma.

### Material and methods

#### Study population

We retrospectively included fifty-seven patients with asthma (30 with severe and 27 with mild to moderate asthma). We excluded tobacco smokers and patients with clinically unstable asthma. The control group comprised 25 healthy, non-smokers volunteers without any respiratory tract infections, four weeks before the study.

Body mass index (BMI) was calculated based on patients' body weight and height (Table 1). World Health Organization (WHO), BMI criteria for adults were used as the values for determining the obesity and overweight.

The diagnosis of asthma was made based on history of asthma symptoms, and reversible airflow limitation, in accordance with GINA 2014 recommendation. We used the ENFUMOSA criteria to assess disease severity [12]. Patients in the group with severe asthma (SA) required continuous treatment with high doses of inhaled corticosteroids ( $\geq 1600 \mu\text{g/day}$  budesonide or beclomethasone,  $800 \mu\text{g/day}$  fluticasone or equivalent). In chronic oral steroid therapy the dosage amounted to  $800 \mu\text{g/day}$  budesonide or beclomethasone,  $400 \mu\text{g/day}$  fluticasone or equivalent. Also patients with severe asthma required long-term therapy, long-acting  $\beta$ -agonist (LABA, long acting  $\beta$ -agonist, formoterol  $9 \mu\text{g/dose}$ , 2 times per day or salmeterol  $50 \mu\text{g/dose}$ , 2 times per day) or oral theophylline ( $200\text{-}300 \text{ mg}$  1-2 times per day). Asthma in these patients has not been fully controlled; in the year before our study these patients had at least one exacerbation.

Patients with mild-moderate asthma (MA) were treated by  $800 \mu\text{g/day}$  of budesonide or beclomethasone,  $500 \mu\text{g/day}$  fluticasone or equivalent. Patients enrolled in the study group did not receive other immunosuppressive medications. In each patient, spirometry, skin prick tests and asthma control test (ACT) were performed. Atopy was ruled in based on positive reaction to at least one allergen [13]. The characteristics of the tested groups are shown in Table 1. An institutional review board approved the study protocol (RNN/133/11/KE), before study initiation. All participants provided written informed consent.

## Experimental procedures

**Blood collection and processing:** Venous blood was drawn into S-Monovette<sup>®</sup> blood collection tubes (Sarsted, German) containing EDTA K and activator of coagulation. Serum was obtained by centrifugation at  $2000 \text{ g}$  for 10 minutes at  $25^\circ\text{C}$ , and stored at  $-80^\circ\text{C}$ . All samples were drawn around 8 AM in the morning, and after 12 h of fasting.

**Adipocytokine measurements of leptin, leptin receptor, adiponectin and resistin:** Concentrations of adipocytokines were measured in serum with ELISA immunoenzymatic tests (leptin - LEPTIN ELISA, DRG Diagnostic, leptin receptor - QUANTIKINE, human leptin sR, R&D Systems, adiponectin - QUANTIKINE, human Adiponectin,

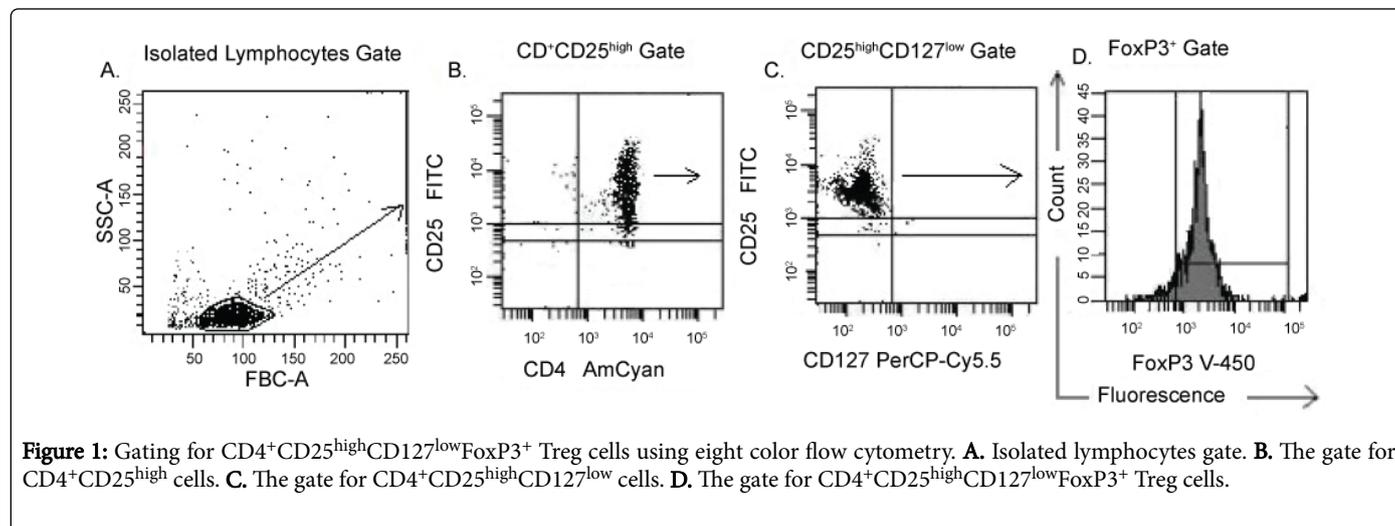
R&D Systems and resistin - QUANTIKINE, human Resistin, R&D Systems). Color intensity was measured spectrophotometrically with ELISA reader ELx800 at the wavelength of  $450 \text{ nm}$ .

**PBMC isolation:** PBMC (peripheral blood mononuclear cells) were purified from EDTA peripheral blood using density gradient centrifugation ( $400 \text{ g}$ ,  $30 \text{ min}$ ) over Histopaque-1077 (Sigma-Aldrich). The percentage of viable isolated cells (PBMC) was determined on Pentra DX 120 analyzer. The purity of the PBMC was more than 80%.

**Isolation of CD4<sup>+</sup>CD25<sup>+</sup> cell subpopulation:** CD4<sup>+</sup>CD25<sup>+</sup> cells were isolated from PBMC using, Human CD4<sup>+</sup>CD25<sup>+</sup> T Cell Isolation Kit (Miltenyi Biotec, USA). The isolation was performed in a two-step procedure. First, non-CD4<sup>+</sup> T cells were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against all types of PBMCs except the CD4<sup>+</sup> T cells. The labeled PBMCs cells were subsequently depleted over a MACS Column. The purity of the enriched CD4<sup>+</sup> T cells was analyzed by flow cytometry ( $95\% \pm 2.7$ ). In the second step, the flow-through fraction of pre-enriched CD4<sup>+</sup> T cells was labeled with CD25 MicroBeads for subsequent positive selection of CD4<sup>+</sup>CD25<sup>+</sup> cells. The purity of the enriched CD4<sup>+</sup>CD25<sup>+</sup> cells was more than ( $93.8\% \pm 2.45$ ).

**Immunostaining and flow cytometry:** Freshly processed CD4<sup>+</sup>CD25<sup>+</sup> cells in FACS buffer were divided into tubes  $1 \times 10^6$  cells per tube. The following monoclonal antibodies were used to stain the FoxP3<sup>+</sup> Treg subpopulations: anti-human CD4 conjugated to Pacific Blue (RPA-T4 clone), anti-human CD25 conjugated to FITC (M-A251 clone), anti-human CD127 conjugated to PE (HIL-7R-M21 clone), and anti-human FoxP3 conjugated to Alexa 647 (259D /C7 clone). All antibodies were purchased from BD Pharmingen. Staining of the superficial antigens was performed according to the manufacturer's procedure (BD Bioscience). Staining of the intracellular transcription factor FoxP3 was performed after the permeabilization of cells, according to the manufacturer's procedure (BD Bioscience).

Cells were acquired using eight-color, three laser, flow cytometer FACS CANTO II (BD Bioscience). Analysis of expression of particular antigens and mean fluorescence intensity measured as mean fluorescence channel (MFC) was performed with the use of FACS DIVA v.6.2 software. A minimum of 15000 CD4<sup>+</sup> events were collected for each sample. The isolated lymphocytes were gated according to Forward Scatter (FSC) and Side Scatter (SSC) parameters (Figure 1A).



The second gate was created for the subpopulation of CD4<sup>+</sup>CD25<sup>high</sup> (Figure 1B). Then, the gates were set around populations CD25<sup>high</sup> vs CD127<sup>low</sup> (Figure 1C). To analyze intracellular FoxP3 expression these cells were gated as CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> events. The expression of FoxP3 was measured within CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> cells (Figure 1D). Automatic compensation for spectral overlap was performed electronically to minimize fluorescence spillover, using antibody capture beads. Gating strategy was determined by comparison to Fluorescence Minus One (FMO) control.

### Statistical analysis

The results were analyzed statistically with the STATISTICA v 10 PL software. Variables were assessed regarding the distribution and equality of variances. Quantitative variables were characterized with median, lower and upper quartile. Comparisons of studied features between groups were performed with Mann-Whitney U-test. Correlations were assessed with the Spearman correlation rank coefficient. Statistical significance was taken as p<0.05.

### Results

Anthropometric data showed that both female and male patients with severe asthma presented higher BMI in comparison to mild-moderate asthma patients and healthy control group (Table 1).

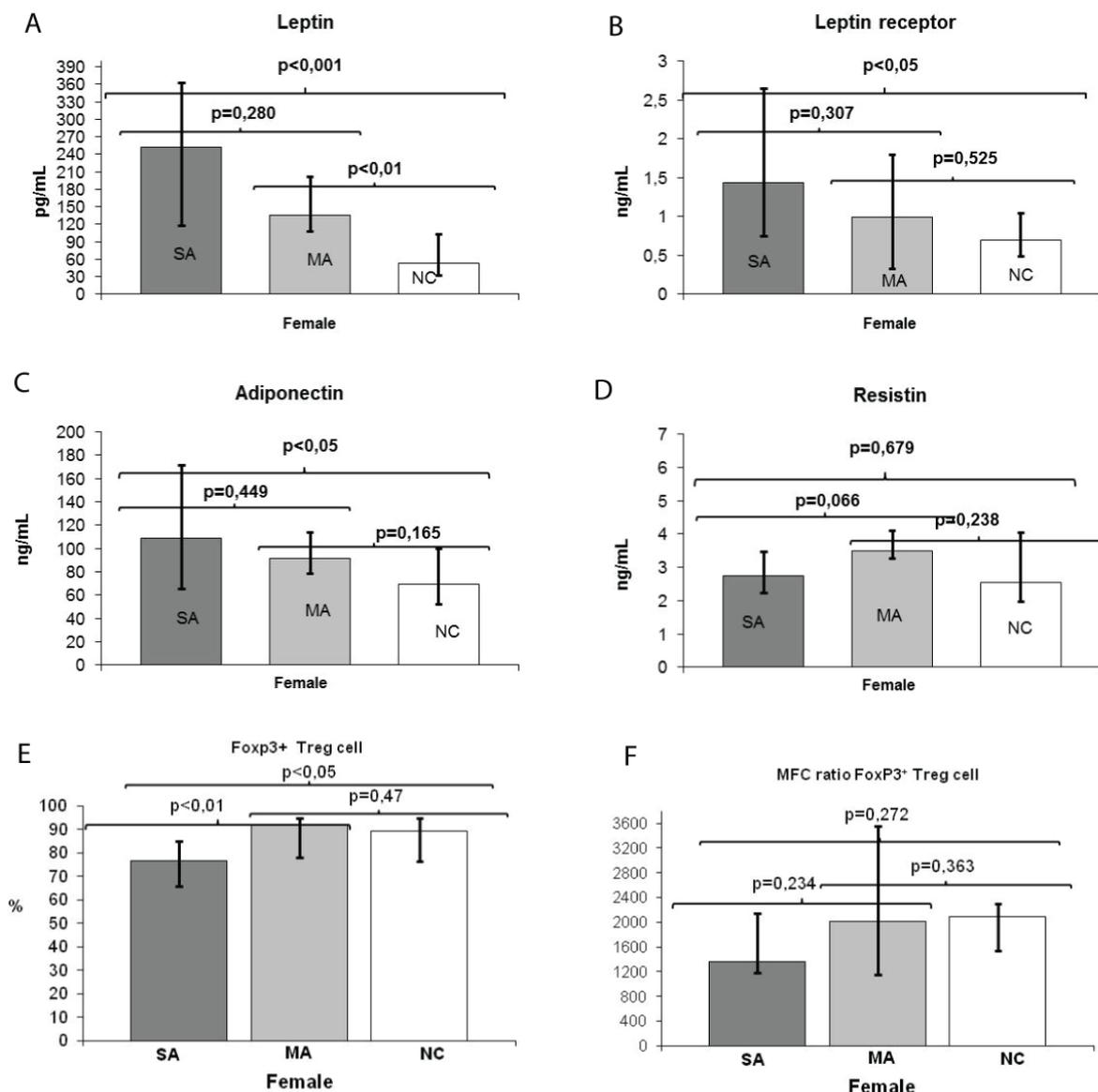
	Severe asthma	Mild-moderate asthma	Control group
N	30	25	27
Sex (F:M)	17:13	13:12	14:13
Age (years, F ± SD; M ± SD)	50 ± 5.5; 48 ± 13.5	43 ± 13.5; 41 ± 12.5	40 ± 11.5; 43 ± 9.5
BMI (F ± SD; M ± SD)	29.2 ± 6.90; 26.5 ± 2.20	22.8 ± 3.90; 24.2 ± 2.80	24.5 ± 1.20; 23.9 ± 1.15
Duration of disease (years, F ± SD; M ± SD)	15 ± 7.5; 17 ± 9.7	9 ± 5.1; 11 ± 6.3	-
Atopy (Y/N; F/M)	14/3; 11/2	11/2; 12/1	0/14; 0/13
FEV <sub>1</sub> (%; F ± SD; M ± SD)	67 ± 15.0; 63 ± 11.2	75 ± 9.5; 80 ± 8.2	92 ± 6.5; 91 ± 5.4
ACT (points, F ± SD; M ± SD)	9.5 ± 2.60; 11.0 ± 3.40	21.5 ± 3.50; 23.5 ± 2.50	-

BMI: Body Mass Index; FEV1: Forced Expiratory Volume; ACT: Asthma Control Test

**Table 1:** Characteristics of the studied group.

	Severe asthma	Mild-moderate asthma	Control group
Leptin (pg/mL)	252.8 (116.7-362.6)	136.0 (107.2-201.5)	56.6 (31.9-102.4)
Leptin receptor (ng/mL)	1.44 (0.74-2.64)	0.95 (0.33-1.79)	0.70 (0.49-1.04)
Adiponectin (ng/mL)	108.5 (64.9-171.3)	91.2 (78.1-113.5)	69.5 (52.1-99.3)
Resistin (ng/mL)	2.74 (2.20-3.45)	3.48 (3.25-4.08)	2.54 (1.97-4.05)
FoxP3 <sup>+</sup> Treg cells (%)	76.5 (66.1-85.2)	91.6 (78.8-94.5)	89.3 (76.6-94.6)
MFC for FoxP3	1354 (1188-2148)	2010 (1153-4502)	2091 (1532-2298)

**Table 2:** Variable (median, lower and upper quartile) describing the leptin, leptin receptor, resistin, adiponectin concentration and percentage of FoxP3<sup>+</sup> Treg cells, mean fluorescence intensity (MFC ratio) for FoxP3 in women group.



**Figure 2:** Comparison of the leptin, leptin receptor, resistin, adiponectin concentration and percentage of FoxP3<sup>+</sup> Treg cells, mean fluorescence intensity (MFC ratio) for FoxP3 between female with severe asthma (SA), mild-moderate (MA) and the control group (NC).

### Correlations in female tested group

We found the positive correlation between leptin concentration and BMI in female with severe asthma (Spearman  $r=0.74$ ;  $p<0.001$ ). We did not observe significant correlation between leptin, or leptin receptor or resistin or adiponectin concentration and MFC ratio. Significant correlations were not found between adipocytokines concentration and the percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup> in women tested group (Table 4).

### Adipocytokines concentration, in male tested group

In male group with severe asthma we observed significantly higher concentration of leptin and leptin receptor than in healthy control group ( $p<0.05$ ) (Figures 3A and 3B and Table 3). There were no

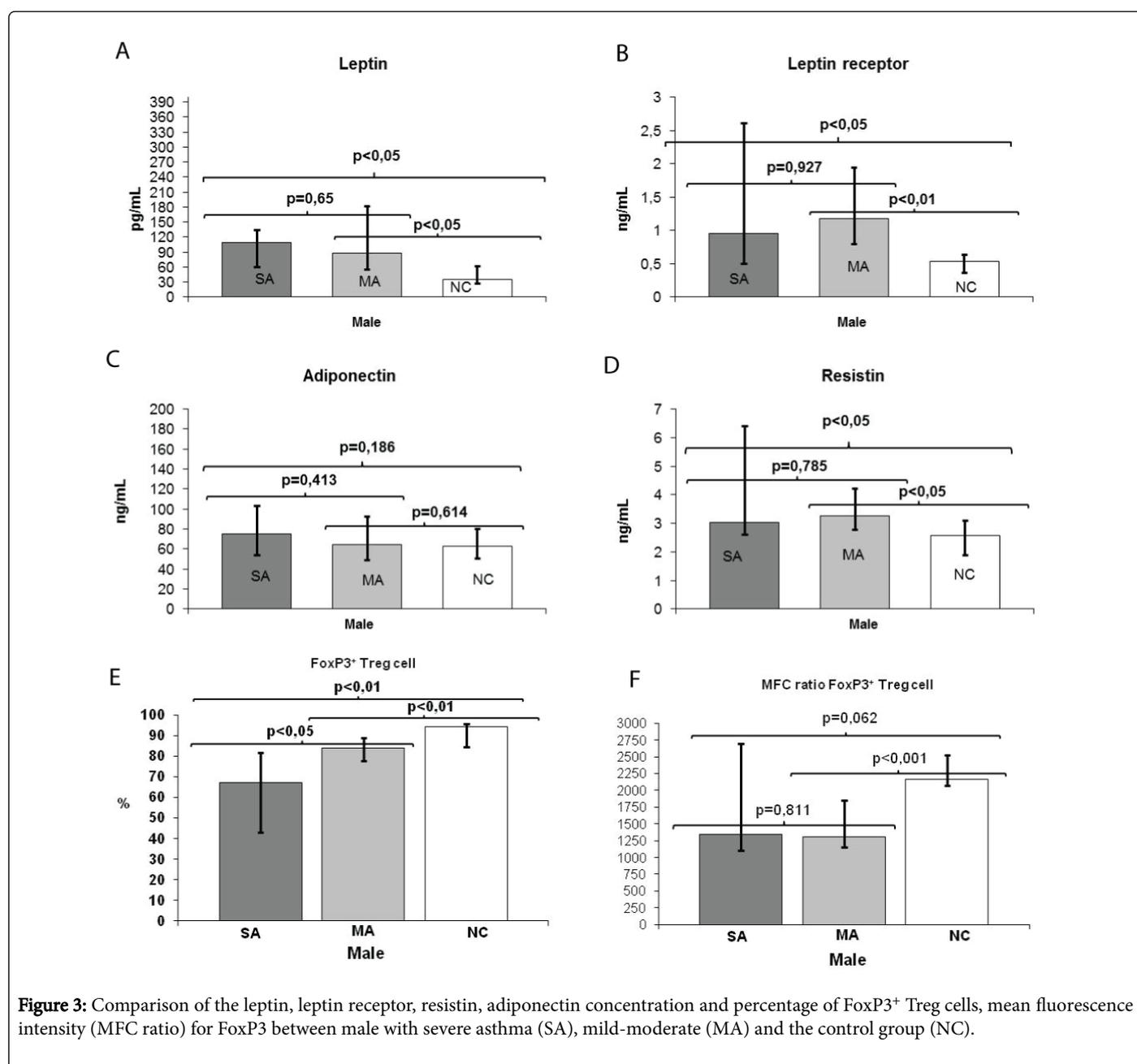
significant differences of adiponectin concentrations in asthmatic male patients compared to healthy control group (Figure 3C and Table 3). The concentration of resistin in male patients with severe asthma was significantly higher than in healthy control group ( $p<0.05$ ) (Figure 3D and Table 3).

### FoxP3<sup>+</sup> Treg cells in male tested group

The percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup> was significantly decreased in male with severe asthma in comparison to mild-moderate ( $p<0.01$ ) and healthy control group ( $p < 0.05$ ) (Figure 3E and Table 3). These significant differences in MFC ratio were observed between male with mild-moderate asthma and healthy control group (Figure 3F and Table 3).

	Severe asthma	Mild-moderate asthma	Control group
Leptin (pg/mL)	108.5 (59.7-134.2)	87.2 (55.2-181.6)	35.5 (26.9-61.8)
Leptin receptor (ng/mL)	0.95 (0.54-2.64)	1.18 (0.79-1.84)	0.54 (0.36-0.63)
Adiponectin (ng/mL)	75.1 (53.7-103.4)	64.4 (48.7-92.3)	62.8 (50.7-80.1)
Resistin (ng/mL)	3.04 (2.63-6.41)	3.27 (2.78-4.27)	2.58 (1.87-3.09)
FoxP3 <sup>+</sup> Treg cells (%)	67.0 (43.5-81.2)	84.5 (77.6-89.4)	94.5 (84.4-95.7)
MFC for FoxP3	1345 (1100-2708)	1309 (1149-1858)	2168 (2077-2523)

**Table 3:** Variable (median, lower and upper quartile) describing the leptin, leptin receptor, resistin, adiponectin concentration and percentage of FoxP3<sup>+</sup> Treg cells, mean fluorescence intensity (MFC ratio) for FoxP3 in men group.



**Figure 3:** Comparison of the leptin, leptin receptor, resistin, adiponectin concentration and percentage of FoxP3<sup>+</sup> Treg cells, mean fluorescence intensity (MFC ratio) for FoxP3 between male with severe asthma (SA), mild-moderate (MA) and the control group (NC).

		Severe asthma		Mild-moderate asthma	
		Female	Male	Female	Male
BMI/Leptin (pg/mL)	r	0.740	0.254	0.411	0.365
	p	0.001	ns	ns	ns
Leptin (pg/mL)/ FoxP3 <sup>+</sup> T <sub>reg</sub> cells (%)	r	-0.284	-0.125	0.110	-0.102
	p	ns	ns	ns	ns
Leptin receptor (ng/mL)/ FoxP3 <sup>+</sup> T <sub>reg</sub> cells (%)	r	-0.395	-0.208	0.115	-0.315
	p	ns	ns	ns	ns
Adiponectin (ng/mL)/ FoxP3 <sup>+</sup> T <sub>reg</sub> cells (%)	r	0.224	0.339	0.315	0.310
	p	ns	ns	ns	ns
Resistin (ng/mL)/ FoxP3 <sup>+</sup> T <sub>reg</sub> cells (%)	r	-0.079	-0.308	-0.211	-0.359
	p	ns	ns	ns	ns
Leptin (pg/mL)/ MFC for FoxP3	r	-0.093	-0.391	-0.421	-0.225
	p	ns	ns	ns	ns
Leptin receptor (ng/mL)/ MFC for FoxP3	r	0.234	0.435	0.372	0.295
	p	ns	ns	ns	ns
Adiponectin (ng/mL)/ MFC for FoxP3	r	0.351	0.046	0.410	0.395
	p	ns	ns	ns	ns
Resistin (ng/mL)/ MFC for FoxP3	r	-0.251	-0.163	0.363	0.286
	p	ns	ns	ns	ns

**Table 4:** Correlation analysis.

### Correlations in male tested group

We did not find significant correlations between leptin or leptin receptor or resistin or adiponectin concentration and MFC ratio. No significant correlations were observed between adipocytokines concentration and the percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup> (Table 4).

### Discussion

Patients with asthma are usually well controlled [14]. Current management of asthma therapy includes corticosteroids with long-acting β<sub>2</sub>-agonists or leukotriene treatment. This therapy leads to good control of disease in most of the patients. There are subpopulations of asthma patients with severe disease whose symptoms and control are believed to be largely unresponsive to treatment, including high-dose inhaled and systemic corticosteroids. These patients with severe asthma suffer greater morbidity, and consume a greater proportion of health resources than other asthmatic patients [15]. Therefore, new therapies still are searched. One of them is inflammatory process modulation by Treg cells.

It has been shown that obesity, especially the visceral type, induces a chronic subclinical inflammatory process [16]. The relationship between asthma and obesity is not fully understood and explained [17]. It has been found that obese asthmatic patients have poor asthma control with severe type of this disease [18]. It is very well known fact

that the concentration of adipocytokines in blood is related to the adipose tissue.

In our study we observed that 70% of female and 50% of male asthmatic patients were overweight. We suggested that the higher BMI in asthmatic patients with severe asthma is induced by high-dose systemic steroid therapy. Furthermore higher BMI in women with asthma is mainly due to the effect of hormonal disturbances according to the age (postmenopausal).

Many data indicate that leptin induces inflammatory processes in asthma. Our results showed significantly higher leptin levels in patients with severe asthma and mild-moderate asthma. We suggested that it was associated with excess weight. Muc et al. also observed that overweight patients with asthma demonstrated higher levels of leptin. It has been suggested that the overweight is the main factor of high leptin concentrations [19]. In women with severe asthma we observed that the concentration of leptin was 5-fold higher. We suggest that it is result of high-dose steroids therapy and postmenopausal age. Our study showed the positive correlation between leptin concentration and BMI in women. The long-term cross-sectional study made by Sood et al. does not confirm this association [20].

There are strong evidences which indicate that leptin influence regulatory T CD4<sup>+</sup> cells. Our results showed that leptin does not directly affect the FoxP3<sup>+</sup> Treg cells. We concluded that leptin may act by different mechanism. Leptin exerts a negative influence on the

proliferative process of human regulatory CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. This adipocytokine put them into a state of anergy [21]. High leptin concentration reduces number and suppress function of Treg cells. These changes lead to the exacerbation of inflammation [22]. Wei et al., demonstrated that leptin inhibits Tregs expression of TGF- $\beta$ , IL-10, CTLA4, and GITR and inhibits function these cells [23].

It is well known fact that adiponectin has anti-inflammatory potential. This adipocytokine suppresses synthesis of proinflammatory cytokines e.g. (TNF- $\alpha$ , IL-6, INF- $\gamma$ ) and development of inflammation [24]. It is suggested that adiponectin concentration is related to the sex and obesity but not related to the asthma [4]. Surprisingly, in our results the concentration of adiponectin was highest in women with severe asthma. This higher concentration of adiponectin in women with severe asthma may suggest loss of anti-inflammatory function. This can contribute in asthma prevalence and severity.

In premenopausal women it has been shown that the high serum adiponectin concentration is associated with low risk of asthma [25]. Experimental study have shown that adiponectin also inhibits the function of T lymphocytes [26].

Resistin is a pro-inflammatory adipocytokine. Our results showed that high resistin concentration was observed only in men with severe and mild-moderate asthma. Larochelle et al. also presented similar results. They also suggested that high resistin concentration directly connected with the asthma severity [27]. It has been shown that high resistin levels predicted favorable anti-inflammatory effect of inhaled glucocorticoids and may be a biomarker of steroid-sensitive phenotype of asthma [28]. It was also found that resistin is responsible for the expansion of regulatory T cells (Treg) through modulation of dendritic cells [29].

We demonstrated that percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup> was decreased in asthma patients. Some reports confirm these results [30]. Surprisingly, decreased percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Treg cells expressing Foxp3<sup>+</sup> was found despite the corticosteroids therapy. Karagiannidis et al. study has shown that glucocorticoid treatment upregulates the mRNA FOXP3 expression. FOXP3 mRNA expression was significantly increased in asthmatic patients who received inhaled or systemic glucocorticoid treatment, or both [31].

In summary, our results confirmed that patients with severe asthma have the pro-inflammatory adipocytokines profile. Surprisingly the concentrations of anti-inflammatory adiponectin were the highest in severe asthma group. We suggested that impaired immunosuppressive activity of Treg cells expressing Foxp3 in patient with severe asthma may result in low number of these cells. In our study we did not confirm the relationship between leptin, adiponectin, resistin and FoxP3<sup>+</sup> Treg cells in patients with severe asthma. Our data suggest that the reduction of body weight in patients with severe asthma can improve the clinical condition by reducing pro-inflammatory adipocytokines.

## Conclusions

- The proinflammatory adipocytokines profile in SA patients and low percentage of FoxP3<sup>+</sup> Treg cells enhances inflammatory response in SA.
- It was not observed the relationship between leptin, adiponectin, resistin and the percentage of CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs expressing Foxp3<sup>+</sup> in patients with severe asthma.

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## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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